

Regulation of cell surface GLUT1, GLUT3, and GLUT4 by insulin and IGF-I in L6 myotubes

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Abstract The effects of insulin and IGF-I on the cell surface quantities of GLUT1, GLUT3 and GLUT4 glucose transporters in L6 myotubes were determined with the exofacial bis-mannose photolabel (ATB-BMPA). In basal cells, an equal molar quantity of each transporter isoform was found at the cell surface. Insulin stimulated the translocation of all three glucose transporter isoforms to the plasma membrane fraction from the light microsome fraction, resulting in equal molar quantities on the cell surface. IGF-I stimulated a similar translocation of all isoforms, augmented by an increase in surface GLUT3 as assessed by ATB-BMPA.

Key words: L6 myotube; GLUT1; GLUT3; GLUT4; Glucose transport; Bis-mannose photolabel

1. Introduction

The L6 cell line has been used to investigate skeletal muscle glucose uptake in response to acute stimulation by insulin or IGF-I. L6 differentiated myotubes were found to contain multiple glucose transporter isoforms [1,2]. These include GLUT1 which is ubiquitously expressed, GLUT3 which is expressed principally in neurons of rodents, and GLUT4 which is expressed in insulin responsive tissues (see reviews [3–5]). Subcellular fractionation demonstrated that GLUT1, GLUT3 and GLUT4 protein levels increase in the plasma membrane-enriched fraction in response to insulin or IGF-I treatment. Acute stimulation with either hormone did not alter mRNA of GLUT1 or GLUT4, nor total protein levels of GLUT1, GLUT3, or GLUT4 [1,2,6,7]. Therefore, the increase in glucose uptake could not be accounted for by hormonal stimulation of glucose transporter protein synthesis but, instead appeared to correspond to a redistribution of transporters. However, due to the differences in antibody immunoreactivity and limitations of membrane fractionation, these techniques cannot be used to make comparisons between the molar ratios of each isoform nor the quantities of functional transporters on the cell surface in control and hormonally stimulated cells.

The impermeant exofacial photoaffinity label 2-N-4-[³H](1-azido-2,2,2-trifluoroethyl)benzoyl-1,3-bis-(D-mannose-4-yloxy)-2-propylamine (ATB-BMPA) has been used to quantitate the functional cell surface levels of GLUT1, GLUT2, GLUT3, and GLUT4 in intact tissues and in cell cultures [8–13]. The aim of this study was to quantitate the functional cell surface levels of

each glucose transporter isoform present in the L6 myotubes in response to insulin and IGF-I.

2. Materials and methods

2.1. Materials

α -Minimal Essential Medium (α -MEM), fetal bovine serum, and other tissue culture reagents were obtained from Gibco (Burlington, Ont.). Human recombinant IGF-I was a gift from Dr. M. Vranic, University of Toronto, Toronto, Canada. Insulin, cytochalasin B, and 2-deoxyglucose were obtained from Sigma (St. Louis, MO). 2-Deoxy-[³H]D-glucose was purchased from ICN (Irvine, CA). Protein-A-Sepharose CL-4B (PAS) was obtained from Pharmacia (Piscataway, NJ). Rabbit polyclonal antisera, specific for the COOH-terminal sequences of GLUT1 (amino acids 472–492), mouse-GLUT3 (amino acids 474–493), and GLUT4 (amino acids 490–509) were kindly supplied by Hoffmann-La Roche (Nutley, NJ). The ATB-BMPA, specific activity of 10 Ci/mmol, was a kind gift from Dr. G.D. Holman, University of Bath, Bath, UK.

2.2. Cell culture

A spontaneously rapid fusing subclone of the original L6 skeletal muscle cell line [14] was grown in monolayer to the stage of 90% fused myotubes as previously described [2]. Cells were deprived of serum for 5 h prior to experimentation using serum free α -MEM supplemented to 25 mM glucose (final concentration). For hormonal stimulation, the medium was replaced with fresh serum-free α -MEM supplemented to 25 mM glucose with or without insulin (100 nM) or IGF-I (10 nM) at 37°C for 30 min prior to the assays.

2.3. 2-Deoxy-[³H]D-glucose uptake

After the hormone treatment, cell monolayers were rinsed with glucose-free HEPES buffered saline (HBS) and glucose uptake was measured in HBS with or without hormone, as previously described using 10 μ M 2-deoxy-[³H]D-glucose (1 μ Ci/ml) for 10 min [6]. The results of specific uptake are expressed as pmol/mg total cell protein/min. Total cell protein/dish was determined by the Bradford method [15].

2.4. Photolabeling

As for the transport assay, after hormonal treatment, the cells were washed three times in HBS solution. ATB-BMPA was added to 1 mCi/ml (100 μ M) in 1 ml HBS with or without hormone for 1 min. Cells were then irradiated for 2 \times 1 min intervals using a Rayonet photochemical reactor (with 300 nM lamps) with gentle rocking between exposures to keep the monolayer moistened. The radioactive medium was aspirated and the cells were immediately solubilized in 1 ml of 2% Thesit (C₃₀H₆₂O₁₀) detergent (Boehringer-Mannheim, Indianapolis, IN) for 30 min at room temperature as previously described [9]. The solubilized cells were scraped off the dish and centrifuged at 14,000 rpm for 10 min to remove insoluble debris. Two 10 ml samples were retained for duplicate protein determination using the bicinchoninic acid assay (Pierce Rockford, IL).

2.5. Immunoprecipitation and quantitation of ATB-BMPA-labeled glucose transporters

Immunoprecipitation was carried out as previously described [12,13] with the following modifications. Thesit-solubilized total L6 protein was first added to 80 μ l of uncoupled 50% PAS (as a preclearing step

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to remove non-specific binding) for 20 min at 4°C. The precleared samples were then transferred to the antibody/PAS immunocomplex and mixed at 4°C for 4 h (for each immunoprecipitation) in the sequential order of GLUT4, GLUT3 then GLUT1. The GLUT3 and GLUT1 immunoprecipitations were carried out twice to maximize efficiency. These procedures gave an efficiency of 85–90% for each isoform. After precipitation, the pellet was washed twice with 1 ml of 0.2% Thesit in PBS followed by 2 × 1 ml PBS. Finally, the immunoprecipitates were eluted from the PAS with sample buffer containing 15 mM Tris, 1.5% SDS, 2.3 M urea, and 100 mM dithiothreitol (final concentrations) for 30 min at room temperature. Solubilized immunoprecipitates were recovered by centrifugation through Millipore 0.45 µm filters in microfuge tubes and loaded on 10% SDS-polyacrylamide gels for electrophoresis. In the cases where 2 rounds of immunoprecipitation were performed, the solubilized complexes were pooled prior to loading on the gels. The gels were cut, solubilized and counted for ³H as previously described [10,13]. The peak corresponding to each transporter was quantitated and expressed as dpm/mg protein for each dish.

2.6. Calculations

A value for total cell surface binding, B_{max} , was derived by the equation $B_{max} = (B)(K_d)((1 + F/K_d)/F)$ with dissociation constants, K_d , previously determined for ATB-BMPA binding to each transporter isoform (GLUT1 = 150 µM, GLUT4 = 150 µM, GLUT3 = 55 µM) [10,12,16,17], the concentration of free ligand ($F = 100$ µM), and the measured bound ligand (B) in pmol/mg total protein, (calculated from the specific activity and total cell surface binding) [17,18]. This calculation accounts for the affinity differences of each glucose transporter isoform for ATB-BMPA. All data are reported as the final (corrected) molar concentrations (pmol/mg total protein), and comparisons were made using these values.

Statistical significance was tested with a one-way analysis of variance followed by a Student-Newman-Keuls or Fisher PLSD post-hoc analysis, where differences were accepted as significant at the $P < 0.05$ level.

2.7. Membrane isolation and Western blotting

L6 myotubes were grown and stimulated with insulin and IGF-I as described above to use for membrane fractionation. Plasma membrane fractions and intracellular light microsome fractions were prepared, equivalent amounts of protein for each membrane fraction were loaded on SDS-polyacrylamide gels and Western blotted using the C-terminus specific antisera, as previously reported [1,6]. Values are reported as relative densitometric units per mg protein.

3. Results

3.1. 2-Deoxy-[³H]D-glucose uptake

Fig. 1 shows the 2-deoxyglucose uptake (pmol/mg protein/min) in unstimulated (basal) cells and after 30 min of insulin- or IGF-I-treatment. Insulin-stimulated uptake was 160% greater than basal ($P < 0.05$) and IGF-I stimulated uptake was 210% greater than basal ($P < 0.01$).

3.2. Cell surface glucose transporters

The cell surface quantities of all three glucose transporter isoforms were determined by photolabeling with the impermeant ATB-BMPA. The specificity of ATB-BMPA was first tested by photoaffinity labeling in the presence or absence of cytochalasin B. The peak of radioactivity for each glucose transporter isoform was reduced by 90% by cytochalasin B (data not shown).

The proportions of cell surface GLUT1, GLUT3 and GLUT4 in basal, insulin and IGF-I treated myotubes are shown in Fig. 2. In basal myotubes there were no statistical differences between the functional quantities of the glucose transporter isoforms at the cell surface (GLUT1 = 1.42 ± 0.20 , GLUT3 = 0.98 ± 0.36 , and GLUT4 = $1.16 \pm 0.24 \times 10^{-2}$ pmol/mg).

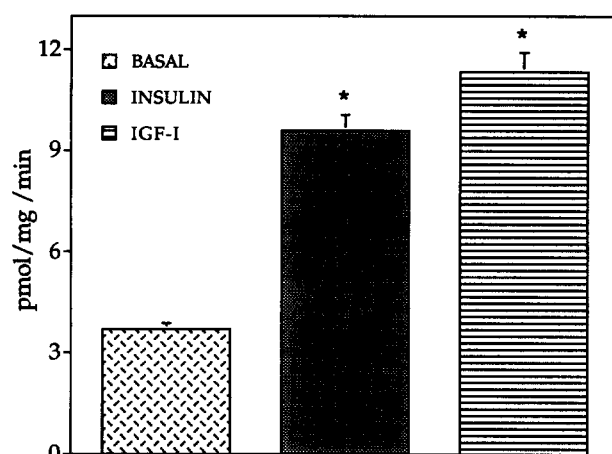


Fig. 1. 2-Deoxy-[³H]D-glucose uptake in L6 myotubes, in basal, 100 nM insulin- and 10 nM IGF-I-treated cells. Hexose uptake was measured after 30 min of hormonal stimulation as described in section 2. Values are mean \pm S.D., $n = 3$. Significance over basal indicated by * at $P < 0.05$.

Insulin stimulation increased cell surface GLUT1 by 16%, GLUT4 by 63% and GLUT3 by 103% above basal levels (Fig. 2). Of these increments, only the GLUT3 was statistically different from basal. There was no statistical difference between the cell surface quantities of GLUT1, GLUT4 and GLUT3 in the insulin-stimulated state.

Relative to basal levels, IGF-I stimulation increased cell surface GLUT1 by 46%, (which was 26% greater than the insulin-stimulated value), GLUT4 by 68% (only 3% greater than with insulin), and GLUT3 by 240% (67% greater than with insulin) (Fig. 2). The cell surface levels of both GLUT4 and GLUT1 in IGF-I treated cells were significantly different from the basal GLUT3 levels. The IGF-I-stimulated increase in cell surface GLUT3 was significantly different from both basal and insulin-treated GLUT3 quantities. This level of surface GLUT3 was also statistically different from IGF-I-stimulated surface GLUT1 and GLUT4, and of all three glucose transporter isoforms at the surface in either the basal or insulin-stimulated myotubes. The change in cell surface GLUT4 in response to IGF-I was not statistically different from basal or insulin-treated GLUT4 levels. GLUT1 surface levels in response to IGF-I were significantly different from the basal state but not significantly different from the insulin-stimulated quantities.

3.3. Subcellular distribution of GLUT1, GLUT3, and GLUT4

Previous studies have demonstrated the translocation of GLUT1, GLUT3 and GLUT4 of L6 cells in response to either insulin or IGF-I. Therefore, plasma membrane-enriched and light microsome-enriched fractions were prepared from cells and Western blotted to confirm that comparable translocation occurred in the present series of experiments.

Insulin stimulation resulted in a 20% increase in GLUT1, a 28% increase in GLUT4, and a 42% increase in GLUT3 in the plasma membrane fraction (Table 1). Concomitant losses of each transporter were observed in the light microsome fraction (GLUT1 decreasing by 44%, GLUT4 by 27%, and GLUT3 by 44%). These results were comparable to the insulin-stimulated translocation shown previously [1,2,6]. IGF-I treatment, in-

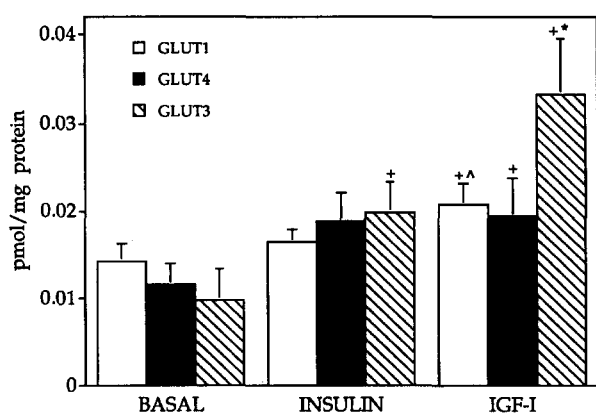


Fig. 2. Cell surface content of GLUT1, GLUT4 and GLUT3 in basal, insulin and IGF-I-treated L6 myotubes. Using ATB-BMPA, cell surface levels of each glucose transporter isoform for each treatment was determined and corrected to pmol/mg total protein as described in section 2. Values are mean \pm S.E.M. ($n = 5-7$). Significance was accepted at the $P < 0.05$ level where: * indicates significance compared to all isoforms under all treatments; ^ indicates significance compared to basal GLUT1; + indicates significance compared to basal GLUT3.

creased plasma membrane levels of GLUT4 by 34% and GLUT3 by 43%, similar to insulin-treatment (Table 1). IGF-I treatment also caused losses from the light microsomal membranes of GLUT1, GLUT4 and GLUT3 (24%, 16%, and 63%, respectively). With the exception of the apparent lack of IGF-I induced changes in plasma membrane GLUT1 in this study, most likely due to a technical difficulty in recovering the fraction, the results here are comparable to the translocation in response to IGF-I shown previously [1,2,6].

Thus, as determined by membrane fractionation, insulin and IGF-I stimulated a similar movement from the light microsomal fraction to the plasma membrane fraction of each isoform. By photolabeling, all three glucose transporter isoforms were present in similar quantities at the cell surface in basal L6 cells. Insulin and IGF-I treatments resulted in comparable increase of GLUT1 and GLUT4 at the cell surface. However, IGF-I treatment increased cell surface GLUT3 to a greater extent than did insulin, and this appeared to exceed the net increase as determined by membrane fractionation.

4. Discussion

The impermeant exofacial photolabel ATB-BMPA binds to glucose transporters and appears to label the active cell surface glucose transporters, or those capable of transporting glucose, and thus is a valuable approach to studying the contributions of individual glucose transporter isoforms to glucose uptake in cells expressing multiple isoforms. The L6 myotubes have been shown to express multiple glucose transporter isoforms and respond to hormonal stimulation by increasing glucose uptake.

Insulin stimulation of L6 cells caused the translocation of all three glucose transporter isoforms, measured as a decrease in the light microsomal content and an increase in the plasma membrane content, as shown previously [1,2,6]. Measurement of the photoaffinity labeled glucose transporters showed that there were similar amounts of each transporter at the surface of L6 cells in the basal state and that, although the increment over basal levels for each isoform differed, similar molar quantities of GLUT1, GLUT3 and GLUT4 were found at the cell surface of insulin-stimulated cells.

The most striking result was the effect of IGF-I treatment on GLUT3 photolabeling. The IGF-I-stimulated 2-deoxyglucose uptake was 3.1-fold greater than basal and 30% greater than insulin-stimulated uptake. The apparent translocation of all three isoforms in response to IGF-I was similar to that seen following insulin-stimulation as described previously [1,2,6]. The increase in cell surface ATB-BMPA labeled GLUT1 and GLUT4 in IGF-I treated cells was similar to that with insulin-stimulation. However, cell surface ATB-BMPA labeled GLUT3 content was greater in IGF-I treated than insulin-treated cells. This indicates that IGF-I stimulation of L6 cells causes a translocation of GLUT1, GLUT4, and GLUT3 similar to that seen with insulin, with a further increase in the accessibility of cell surface GLUT3 to ATB-BMPA.

Using the same methods employed here, the presence of both active and inactive transporters in adipocyte plasma membranes was proposed previously. Glucose transport activity correlated most closely with the amount of GLUT4 photolabeled with ATB-BMPA at the cell surface rather than with the total number of GLUT4 transporters present in the plasma membrane fraction as determined by Western blotting [19,20]. Thus, transporters may be present at or in the plasma membrane in an active state, accessible to glucose or the photolabel,

Table 1
Glucose transporters in membrane fractions (relative units/mg protein)

	Plasma membranes			Light microsomes		
	Basal	Insulin	IGF-I	Basal	Insulin	IGF-I
GLUT 1						
Relative to basal PM	1.00	1.20	1.03	0.38	0.21	0.29
S.D.	0.08	0.18	0.08	0.07	0.03	0.08
GLUT 4						
Relative to basal PM	1.00	1.28	1.34	0.51	0.37	0.43
S.D.	0.17	0.21	0.23	0.09	0.16	0.12
GLUT 3						
Relative to basal PM	1.00	1.42	1.43	0.47	0.19	0.17
S.D.	0.11	0.33	0.33	0.05	0.08	0.09

The distribution of each transporter isoform in plasma membrane and light microsomal membrane fractions made from basal and hormone-stimulated cells is shown. The values are given relative as densitometric units/mg protein (mean \pm S.D., $n = 3$), where the basal plasma membrane fraction value for each isoform was set equal to 1.00.

and an inactive state (or occluded) which was inaccessible to ATB-BMPA. If this is also the case for GLUT3 then the additional cell surface GLUT3 in IGF-I treated cells, which exceed the relative movement to the plasma membrane fraction, would suggest that IGF-I somehow increases (or 'activates') the number of cell surface accessible GLUT3 glucose transporters in the plasma membrane. This might account for the higher glucose transport activity in the presence of IGF-I as compared to insulin.

To correlate glucose transporter number with glucose transporter activity, when more than one glucose transporter isoform is present, the following parameters need to be considered: (1) the relative abundance and accessibility to glucose of each isoform on the cell surface; (2) the affinity of each isoform for the substrate at the concentrations used to measure transport; and (3) the turnover number for each isoform. The relative abundance of GLUT1, GLUT3 and GLUT4 at the cell surface of L6 myotubes has been presented in this report. Current data suggests that GLUT1 has a low affinity (K_m 10–20 mM), while GLUT 4 and GLUT3 have higher affinities (GLUT4, 1.8–7 mM and GLUT3, 3–10 mM) [4,17,21]. Assuming that the turnover number and V_{max} for all three isoforms are similar, and the K_m is as listed above, then our data suggests that GLUT3 and GLUT4 account for glucose uptake in basal and insulin-stimulated cells, while GLUT3 probably accounts for the further elevation in glucose uptake with IGF-I treatment.

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